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The Bunyamwera Virus Nonstructural Protein NSs Inhibits Viral RNA Synthesis in a Minireplicon System

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The small (S) genomic segment of Bunyamwera virus (family *Bunyaviridae,* genus *Bunyavirus*) encodes the nucleocapsid protein, N, and a nonstructural protein, NSs, in overlapping reading frames. In order to elucidate the function of NSs, we established a plasmid-based minireplicon system using mammalian cells that express large amounts of T7 RNA polymerase. Expression of N, the viral polymerase protein (L), and a minireplicon containing a reporter gene was sufficient to reconstitute functional virus nucleocapsids. Coexpression of NSs, however, led to a dose-dependent decrease in reporter activity without affecting expression of controls. The inhibition could not be reversed by overexpression of N, L or the minireplicon, indicating that the NSs effect was not caused by a reduction in virus gene expression. The NSs proteins of two other members of the *Bunyavirus* genus, Guaroa virus and Lumbo virus, were also inhibitory in our system. The intracellular localisation of *Bunyamwera* virus NSs was investigated and found to be predominantly cytoplasmic, but intranuclear inclusion was also detected. Taken together, these data suggest that, in mammalian cells, the bunyavirus NSs protein controls the activity of the viral polymerase by a highly conserved mechanism.

Key Words: bunyavirus; negative-stranded RNA virus; nonstructural protein NSs; minireplicon; polymerase repressor.

INTRODUCTION

Most viruses express nonstructural proteins in addition to the structural proteins that constitute the virus particles. Frequently, these cell-associated proteins are not essential for virus replication in cell culture, defining them as accessory proteins, and their function is poorly understood. For viruses of the *Bunyaviridae* family, the role of the nonstructural protein NSs has yet to be established. Members of the *Bunyaviridae* are enveloped viruses and have been classified into five genera: *Bunyavirus, Hantavirus, Nairovirus, Phlebovirus,* and *Tospovirus.* More than 300 individual viruses, transmitted mainly by arthropods, are contained within the family (Elliott, 1996). All members have a trisegmented single-stranded RNA genome of negative or ambisense polarity, replicate in the cytoplasm, and bud into the Golgi apparatus (Bishop, 1996). They encode four common proteins: the viral nucleocapsid protein (N) on the smallest (S) segment, two glycoproteins (G1 and G2) on the medium (M) segment, and the viral polymerase (L) on the large (L) segment. Viruses within some genera also encode nonstructural proteins, either on the M segment (termed NSm) or on the S segment (NSs). For the NSs protein, the strategy of expression differs between genera, and the primary sequences and molecular weights are highly variable, such that there is no evidence for isofunctionality of NSs proteins from viruses in different genera. In addition, members of the *Hantavirus* and *Nairovirus* genera do not possess an NSs gene. For both of these virus groups the N protein is considerably larger than that encoded by the NSs-expressing viruses, and Simons *et al.* (1992) have proposed that the NSs-associated function may be contained within the larger N proteins of these viruses.

The *Bunyavirus* genus includes important pathogens such as La Crosse virus, which is the most common cause of mosquito-borne encephalitic illness in the United States, and Oropouche virus, the cause of an acute febrile illness that constitutes an emerging public health problem in South America (Elliott, 1997). For Bunyamwera virus (BUN), the prototype of both the *Bunyaviridae* family and the *Bunyavirus* genus, NSs is a small hydrophobic protein of 101 amino acids expressed from an internal +1 reading frame within the N gene.

The general features of BUN transcription and RNA replication are similar to those of other negative-stranded RNA viruses (Bishop, 1996). The three genomic RNA segments are encapsidated by N protein and associate with minor amounts of L protein both intracellularly and in the virion, and it is only the nucleocapsids that are functional templates for mRNA synthesis and RNA replication by the viral polymerase.

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Here, we investigated the regulatory role of the BUN NSs protein in RNA synthesis, using a novel *in vivo* system to reconstitute BUN nucleocapsids from cloned cDNAs. The system involves the viral proteins L and N and a bunyavirus-like minireplicon that serves as a reporter for the reconstituted BUN polymerase activity. We were able to show that NSs is a potent repressor of the BUN polymerase in our system. Furthermore, we found that NSs is expressed mainly in the cytoplasm, the subcellular compartment for virus replication.

RESULTS

Effect of NSs protein expression on the activity of the BUN minireplicon system

Previously we described the intracellular reconstitution of BUN nucleocapsids from transiently expressed components supplied on plasmids (Dunn *et al.,* 1995). The minimal components of this minireplicon system were (i) plasmid expressing BUN L, the virus polymerase; (ii) plasmid expressing BUN N, the viral nucleocapsid protein; and (iii) minireplicon RNA, an analog of viral genomic RNA consisting of a negative-sense chloramphenicol acetyltransferase (CAT) reporter gene flanked by BUN promoter sequences. The L and N proteins were shown to be both necessary and sufficient to reconstitute active nucleocapsids, leading to expression of CAT enzyme activity. Furthermore, a difference in the level of CAT activity was noted when NSs was coexpressed, but this was not pursued further (Dunn *et al.,* 1995).

In the above system, transcription of BUN sequences from plasmids was driven by bacteriophage T7 RNA polymerase provided by a vaccinia helper virus, vTF7-3 (Fuerst *et al.,* 1986). While this vaccinia helper virus has many advantages such as cytoplasmic capping activity and high levels of expression, it has several drawbacks including the shut-off of nuclear transcription and cytopathic effects. Recently, noncytopathic Sindbis virus replicons (T7-SIN) that allow long-term expression of T7 RNA polymerase in BHK cells have been developed (Agapov *et al.,* 1998). We modified our reconstituted system to express a BUN minireplicon in these T7-SIN cells. For this purpose, the BUN L gene (coding for the polymerase) and the BUN S gene (coding for both N and NSs proteins) were cloned into an internal ribosome entry site (IRES)-containing vector, pTM1 (Moss *et al.,* 1990), allowing translation in a cap-independent manner. Further, plasmids containing the reading frames of N only (with point mutations that abrogate NSs translation) and NSs only were generated. As a reporter we chose *Renilla* (sea pansy) luciferase (REN-Luc). This allowed us to assay reconstituted BUN transcription, by REN-Luc activity, together with *Photinus* (firefly) luciferase (FF-Luc) activity. FF-Luc was expressed from a pTM1 construct and served as an internal control for transfection efficiency and cell viability.

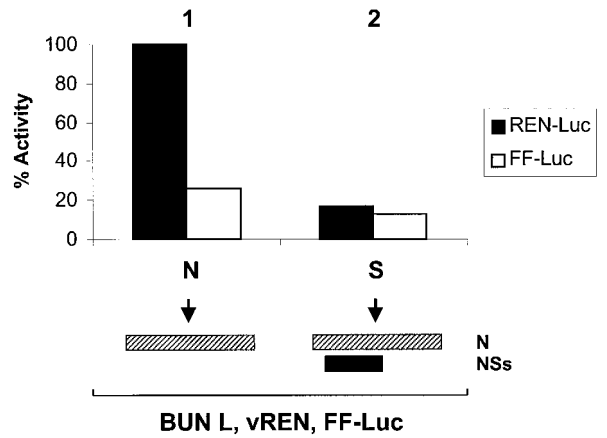

FIG. 1. Expression of a reporter minireplicon by recombinant Bunyamwera virus proteins. T7-SIN cells were transfected with pTM1-BUNL (1 μg), pT7riboBUNMREN(−) (0.5 μg), and either pTM1-BUNN or pTM1-BUNS (0.5 μg), designated here as L, vREN, N, and S, respectively. As an internal control, 0.1 μg of the T7-driven firefly luciferase construct pTM1-FF-Luc was cotransfected. After incubation overnight, cells were lysed and luciferase activities were measured in a dual reporter assay (Promega). *Renilla* luciferase activity (REN-Luc, black columns) reflects minireplicon expression by recombinant BUN proteins. Firefly luciferase activity (FF-Luc, grey columns) reflects expression of the T7-driven control plasmid. The luciferase counts (REN-Luc and FF-Luc) were normalised with respect to the REN-Luc activity of the experiment with pTM1-BUNN in the plasmid mixture. In this figure and the following figures, data from a representative experiment are shown. To clarify differences between the BUN N and the BUN S expression constructs, the respective translation products are depicted below the graph.

As a first step towards the characterisation of the system, we compared the efficiency of minireplicon transcription by BUN L when either BUN N or BUN S (encoding both BUN N and BUN NSs) plasmids were used. The system was optimised by titration of N or S plasmid against constant amounts of L plasmid (data not shown). As shown in Fig. 1, use of pTM1-BUNN resulted in approximately fivefold higher reporter activity than when pTM1-BUNS was used (columns 1 and 2, REN-Luc). To exclude nonspecific inhibition of the T7-SIN system, we showed that the internal control, FF-Luc, was not affected to such an extent when cotransfected into the cells (Fig. 1, columns 1 and 2, FF-Luc). In Fig. 1 and the following figures, the luciferase counts (REN-Luc and FF-Luc) were normalised with respect to the REN-Luc activity of the experiment with pTM1-BUNN in the plasmid mixture.

It was thought possible that the reduced efficiency of the BUN minireplicon system, observed when using the BUN S plasmid, was due to an inhibitory effect of the NSs protein expressed from the BUN S mRNA. Alternatively, it could be that a reduced amount of N was expressed when NSs was translated from the same mRNA and that the larger amounts of BUN S plasmid required to compensate for this would overload the transfection capacity of the system. To distinguish between these possibilities,

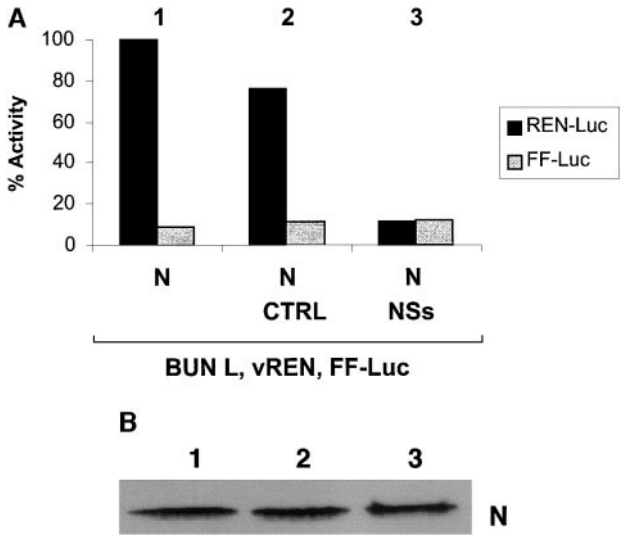

FIG. 2. Effect of NSs in *trans*. (A) T7-SIN cells were transfected with plasmids pTM1-BUNL (1 μg), pT7riboBUNMREN(−) (0.5 μg), and pTM1-BUNN (0.5 μg), encoding the basic components of the BUN minireplicon system, and the FF-Luc internal control (0.1 μg) as described in the legend to Fig. 1. Cells in dish 2 were cotransfected with the control construct pTM1-CTRL (0.5 μg) and cells in dish 3 with the NSs-expression construct pTM1-BUNNSs (0.5 μg). FF-Luc and REN-Luc activities were determined and normalised to the REN-Luc activity of the experiment with no additional plasmid (dish 1). (B) Detection of N protein. Ten micrograms of total cell protein from the above dishes was separated by SDS–PAGE and subjected to Western blot analysis using an anti-BUN N antiserum.

the basic BUN minireplicon system (consisting of BUN L and N proteins and the minireplicon) was supplemented with NSs provided on a separate plasmid. As seen in Fig. 2A, NSs supplied *in trans* (columns 3, REN-Luc) had an inhibitory effect on the BUN minireplicon system similar to that observed when the BUN S construct was used (see above). In contrast, a control construct, pTM1-CTRL, encoding a polypeptide of similar size as NSs (Fig. 2A, columns 2, REN-Luc) did not have such a marked effect (Fig. 2A, columns 1; REN-Luc). Again, there was no significant effect on the internal control (Fig. 2A, columns 1, 2, and 3, FF-Luc).

Although NSs was able to exert its effect when expressed from a separate plasmid, NSs could still have affected BUN N expression in the cotransfected cells. We therefore evaluated N protein levels in cells coexpressing NSs by Western blot analysis. As can be seen in Fig. 2B, N expression levels did not differ between dishes transfected with either the basic plasmid mixture containing pTM1-BUNN (lane 1) or with the addition of NSs (lane 3) or control protein (lane 2).

Taken together, these data suggest that NSs specifically represses the activity of reconstituted BUN polymerase in the minireplicon system.

Subcellular localisation of BUN NSs

There are no reports on the subcellular localisation of the NSs protein encoded by bunyaviruses. Analysis of this would be facilitated by the availability of anti-NSs antiserum, but attempts to produce anti-peptide NSs antisera have so far been unsuccessful (unpublished results). Therefore, in order to determine where BUN NSs locates in the cell, we fused a coding sequence for an N-terminal FLAG peptide to the NSs cDNA sequence. Importantly, the modification did not alter the ability of NSs to repress BUN polymerase activity in the minireplicon system (data not shown), indicating that this activity of NSs was not impaired. Cells were transfected with the FLAG-NSs plasmid and analysed by confocal immunofluorescence microscopy. Figure 3A shows that NSs is located in the cytoplasm of transfected cells. Interestingly, in some cells NSs was also detected in the nucleus, forming aggregate-like structures (Fig. 3B). These data show that the BUN NSs protein is mainly confined mainly to the cytoplasm but can also enter the nucleus.

Overexpression of BUN nucleocapsid components

We further elucidated the possible mechanism by which BUN NSs affects BUN polymerase activity. Individual components of the BUN minireplicon system were overexpressed by increasing the amount of plasmid DNA transfected to see whether the effect of NSs could be outcompeted. Figure 4A (columns 2 to 5) shows that neither the overexpression of BUN N or L proteins, nor of the minireplicon was able to neutralise the NSs-mediated inhibition of the basic minireplicon system (Fig. 4A, columns 1). Figure 4B shows a Western blot that demonstrates that increasing the amount of pTM1-BUNN DNA transfected results in increased experession of N protein; similar results were obtained when L protein expression was analysed (data not shown). The observed inhibition of REN-Luc activity was not due to overexpression of any of the components, however, since replacing the NSs construct with that encoding the control polypeptide restored basic activity or even increased it (Fig. 4A, columns 6 to 9). We also investigated whether BUN NSs could discriminate between the promoters of the three genomic segments. To this end, minireplicon reporter constructs representing the S or L genomic segments of BUN were used in parallel to the M-promoter construct used in the previous experiments. In all cases, a similar degree of inhibition was achieved when NSs was coexpressed (data not shown). Taken together, these results indicate that NSs does not directly inactivate the N or L proteins or the viral genome and inhibits transcription/replication of all BUN segments similarly.

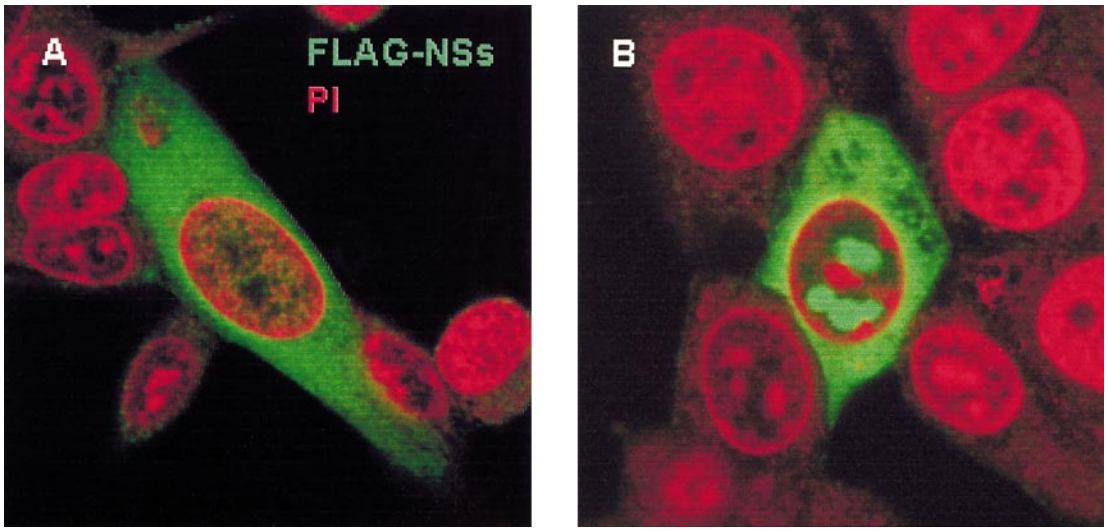

FIG. 3. Subcellular location of BUN NSs. T7-SIN cells were transfected with pTM1-FLAG-NSs, encoding BUN NSs fused to an N-terminal FLAG peptide tag. Cells were fixed 18 h posttransfection and analysed by indirect immunofluorescence using antibodies directed against the FLAG portion of the fusion protein (green). Cell nuclei were counterstained with propidium iodide (PI, red). (A) Cytoplasmic location of FLAG-NSs (green). (B) Cytoplasmic and nuclear location of FLAG-NSs.

NSs acts in a dose-dependent manner

We titrated the amount of NSs plasmid necessary to inhibit the BUN minireplicon system. For this purpose, increasing concentrations of pTM1-BUNNSs were used in the minireplicon system (Fig. 5, columns 2 to 5, REN-Luc). A clear inhibitory effect on REN-Luc activity was evident when cells were treated with as little as 60 ng of NSs plasmid (Fig. 5, columns 2, REN-Luc). This effect was further enhanced with increasing amounts of NSs plasmid (Fig. 5, columns 3 to 5, REN-Luc). In contrast, the control plasmid did not have a significant effect on the system (Fig. 5, compare columns 1 and 6, REN-Luc). Therefore, NSs can exert its inhibitory effect at low plasmid concentrations, suggestive of a high specificity.

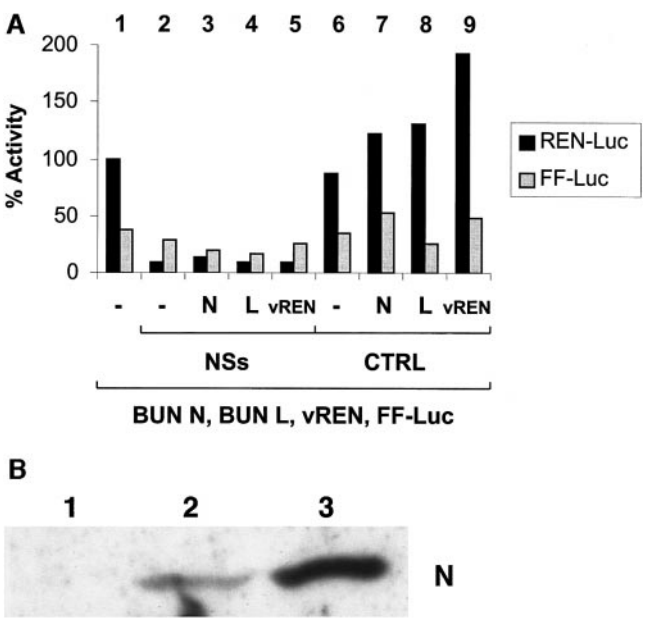

FIG. 4. Overexpression of BUN nucleocapsid components. (A) T7-SIN cells were transfected with plasmids encoding the basic components of the BUN minireplicon system and the FF-Luc control as described in the legend to Fig. 2. Cells in dishes 2 to 5 were cotransfected with pTM1-NSs (0.5 μg) and cells in dishes 6 to 9 were cotransfected with pTM1-CTRL (0.5 μg). In addition, 1 μg extra of pTM1-BUNN was added to dishes 3 and 7, 1 μg extra of pTM1-BUNL to dishes 4 and 8, and 1 μg extra of pTM1-pT7riboBUNMREN(−) to dishes 5 and 9. (B) Detection of N protein in cells transfected with 0 μg (lane 1), 0.5 μg (lane 2), or 1.5 μg (lane 3) pTM1-BUNN DNA. Equal amounts of cell extract were separated by SDS–PAGE and subjected to Western blot analysis using an anti-BUN N antiserum.

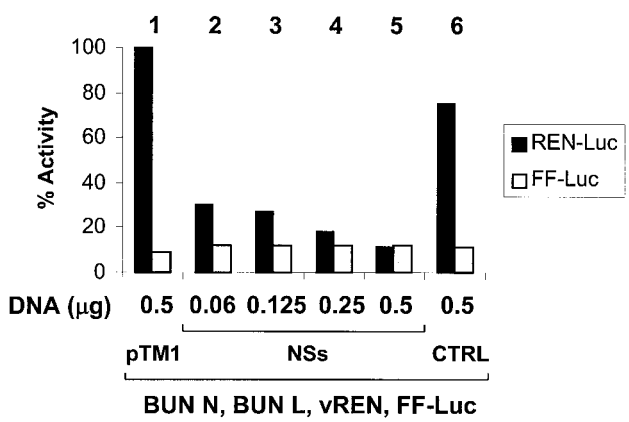

FIG. 5. Concentration dependency of the NSs-mediated inhibition. T7-SIN cells were transfected with plasmids encoding the basic components of the BUN minireplicon system and the FF-Luc control as described in the legend to Fig. 2. In addition, 0.5 μg of pTM1 (dish 1) or pTM1-CTRL (dish 6) was added to the plasmid mixture or increasing amounts of pTM1-BUNNSs as indicated (dishes 2 to 5). Total DNA concentrations were kept constant by addition of the vector plasmid pTM1.

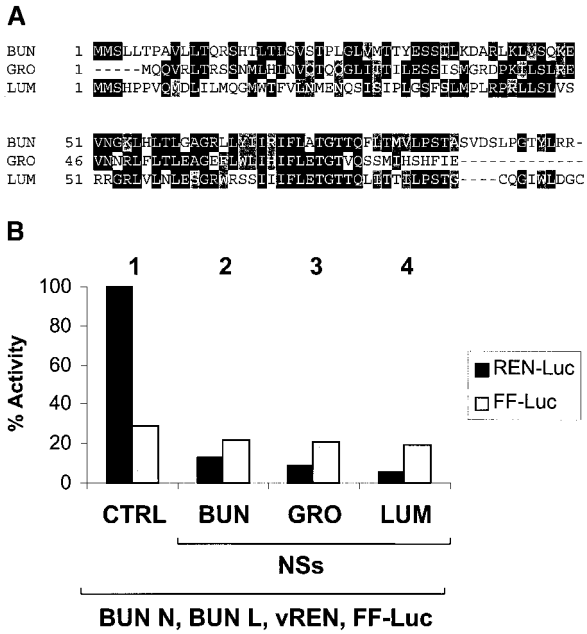

FIG. 6. Effect of NSs proteins from other bunyaviruses. (A) Comparison of the BUN, Guaroa (GRO), and Lumbo virus (LUM) NSs amino acid sequences. Sequence alignments were performed using the CLUSTALW program (Thompson *et al.,* 1994) with default parameter settings. Amino acid residues either identical or with similar chemical properties in at least two of three sequences are shaded in black or grey, respectively. (B) Cells were transfected with plasmids encoding the basic components of the BUN minireplicon system and the FF-Luc control as described in the legend to Fig. 2. In addition, cells were transfected with 0.5 μg of pTM1-CTRL (dish 1), 0.5 μg of pTM1-BUNNSs (dish 2), 0.5 μg of pTM1-GRONSs (dish 3), or 0.5 μg of pTM1-LUMNSs (dish 4).

NSs proteins of other members of the *Bunyavirus* genus

The NSs proteins of bunyaviruses vary in their primary sequences, but within genera contain conserved sequence elements (Elliott, 1996). To investigate a possible role for these elements in the regulation of the reconstituted BUN polymerase, we examined the effect of NSs proteins from other members of the *Bunyavirus* genus in our minireplicon system. NSs from two viruses were studied: Guaroa virus (GRO), which has the shortest and most distant NSs sequence within the Bunyamwera serogroup (Dunn *et al.,* 1994), and Lumbo virus (LUM), a member of the California encephalitis serogroup. The NSs sequences of these viruses vary by 40% (GRO) and 30% (LUM) amino acid identity from BUN NSs, but conserved regions can be identified (Fig. 6A). We studied the activity of these different NSs proteins in the BUN minireplicon system. Interestingly, both GRO NSs and LUM NSs had an inhibitory effect similar to that of BUN NSs (Fig. 6B). This finding suggests that the repressor activity against the viral polymerase is a conserved feature of the NSs protein and can act across virus species boundaries within the *Bunyavirus* genus.

DISCUSSION

This study demonstrates that the bunyavirus nonstructural protein NSs down-regulates the viral polymerase in a minireplicon system that reconstitutes bunyavirus nucleocapsids from transfected cDNAs. NSs was found to be highly active, with input plasmid concentrations of 60 ng per dish resulting in approximately 60% reduction of activity (Fig. 5). In infected mammalian cells, BUN NSs is readily detected by 6 h postinfection, and expression levels parallel those of the N protein, which is translated from the same mRNA (Scallan and Elliott, 1992). That NSs is active at low concentrations and also in the natural context of the S segment gene expression (Fig. 1) suggests that the effect is an authentic one and could also operate during BUN infection.

We ruled out certain trivial possibilities for the inhibitory effect of NSs. Inhibition depended on the presence of an intact NSs ORF, since mutations of the NSs start codons in an otherwise unaltered BUN S expression construct abolished the NSs effect (Fig. 1). This provides evidence that the effect was mediated by the NSs protein rather than the RNA. The synthesis of N protein and of a second reporter protein, both expressed by T7 polymerase, was largely unaffected by NSs (Fig. 2). In some experiments, a reduction (up to 20%) in expression of the internal control could be observed, but this is most probably due to toxic side effects of prolonged NSs expression in the cell and clearly different from the specific suppression of the BUN polymerase activity. This view is supported by the fact that by increasing the expression of nucleocapsid components, which should more than correct for reduced expression, the NSs-mediated inhibition could not be outcompeted (Fig. 4). Thus, the effect of NSs on BUN minireplicon transcription cannot be ascribed to perturbation of plasmid transfection, synthesis of T7 polymerase, T7-mediated transcription, or translation. Furthermore, NSs affects the promoters of all three BUN segments similarly (data not shown). Therefore, it is possible that NSs inhibits a basic activity of the viral polymerase, either by direct interaction or by competition for cellular cofactors.

We also tested NSs proteins of two other members of the *Bunyavirus* genus, Guaroa virus and Lumbo virus, and found that they were inhibitory to the BUN polymerase (Fig. 6), indicating a highly conserved mechanism that can act across bunyavirus species boundaries. Interestingly, segment reassortment between compatible viruses of the *Bunyavirus* genus occurs exclusively early in the multiplication cycle (Iroegbu and Pringle, 1981). Since NSs accumulates in the cell during infection (Scallan and Elliott, 1992) and is present in the cytoplasm, the compartment entertaining virus replication (Fig. 3), it is possible that the high levels of NSs achieved later in infection might suppress growth of a second bunyavirus in the same cell.

The NSs of RVFV, a member the *Phlebovirus* genus, had no such negative effect in an analogous minireplicon system (Lopez *et al.,* 1995). The NSs proteins of viruses in these two *Bunyaviridae* genera, however, are different in size, primary sequence, and in how they are encoded, suggesting functional diversity. It is therefore possible that the NSs proteins of viruses in the different *Bunyaviridae* genera have adopted different or only partially overlapping functions with respect to the viral replication cycle. In this regard it is noteworthy that recent results indicate that both the BUN and the RVFV proteins designated NSs play a role in antagonsim of the interferon response (Bridgen *et al.,* 2001; Haller *et al.,* 2000).

Precedents for proteins with roles as negative regulatory factors can be found in other negative-strand viruses. The M1 (matrix) protein of influenza A virus and the Z protein of lymphocytic choriomeningitis virus have an inhibitory effect on their virus polymerase activity (Watanabe *et al.,* 1996; Lee *et al.,* 2000). The NS1 and M2-2 proteins of respiratory syncytial virus (RSV) have been shown to be potent inhibitors of viral transcription and replication (Atreya *et al.,* 1998; Collins *et al.,* 1996). Similarly, the V and C proteins of SeV and the V protein of measles virus were shown to interfere with genome replication (Curran *et al.,* 1991; Cadd *et al.,* 1996; Tober *et al.,* 1998). Interestingly, the coding strategy of the SeV C protein resembles that of NSs in that it is translated from an alternative reading frame within the ORF for a structural protein (SeV P). This strategy may ensure a constant ratio between the inhibitory protein (SeV C/BUN NSs) and the structural protein (SeV P/BUN N), indicating a functional relationship between these proteins. Indeed, it has been shown that the SeV C protein directly binds to the viral polymerase L, of which the P protein is a subunit (Horikami *et al.,* 1997). Therefore, it is tempting to speculate that NSs has a similar mechanism of action, and attempts to identify a viral interacting protein partner are in progress.

Elucidation of the mechanism of inhibition by intrinsic viral polymerase repressors such as BUN NSs and identification of the viral target structure can help virologists to understand further the intracellular multiplication of negative-stranded viruses and may lead to novel therapeutic and preventive strategies for the control of their associated diseases.

MATERIALS AND METHODS

Plasmid constructions

All plasmids were constructed by using standard molecular biology techniques (Ausubel *et al.,* 1993) and checked by restriction analysis and/or sequencing. The protein-expression plasmids pTM1-BUNS, pTM1-BUNN, pTM1-BUNNSs, pTM1-GRONSs, and pTM1-LUMNSs contained the appropriate coding sequences under control of a T7 promoter and the encephalomyocarditis virus IRES in the vector pTM1 (Moss *et al.,* 1990). The control construct pTM1-CTRL contained the coding sequence for a polypeptide of the same length as NSs. The plasmids were constructed as follows. The ORF was amplified by PCR with an upstream primer containing a *Bsm*BI restriction enzyme site designed to form a *Nco*I overhang upon restriction digestion. The downstream primer contained an *Xho*I restriction enzyme site. Primer sequences are available from the authors upon request. The PCR products were digested with *Bsm*BI and *Xho*I and cloned into *Nco*I–*Xho*I digested pTM1. The template for PCR amplification of BUN S and BUN NSs sequences was pTFBUNS (Dunn *et al.,* 1995). BUN N was amplified from pTZBUNS(−NSs), a construct that contains point mutations that abrogate NSs translation but do not alter the N amino acid sequence (Dunn *et al.,* 1995). To generate expression plasmids pTM1-GRONSs and pTM1-LUMNSs, GRO NSs and and LUM NSs sequences were amplified from the respective pTZ plasmids (Dunn *et al.,* 1994). For the insert of pTM1-CTRL, the 5′ 318 nt of the MxA reading frame were amplified from pBS-T7/MxA (Weber *et al.,* 2000). To generate the N-terminal fusion protein expression plasmid pTM1-FLAG-NSs, the coding sequence for the FLAG epitope (Hopp, 1988), preceded by a translational start codon, was included in the 5′ PCR primer sequence and the ATG start codon of the template reading frame was omitted.

Construction of the pTM1-BUNL plasmid required amplification of an internally deleted L segment cDNA that lacked the sequences between nucleotides 699 and 5660 (Bridgen and Elliott, 1996). The PCR product was cloned into pTM1 using the same strategy as described above. The full-length L ORF was reconstructed by digesting this intermediate construct with *Bsg*I and *Pme*I and inserting the *Bsg*I–*Pme*I fragment (representing L nucleotides 78 to 5947) from pT7riboBUNL (Bridgen and Elliott, 1996).

The internal control plasmid pTM1-FF-Luc was constructed by digesting the *Photinus* (firefly) luciferase expression plasmid pGL3-control (Promega) with *Nco*I and *BamH*I and inserting the cDNA fragment into *Nco*I–*BamH*I-digested pTM1.

The BUN reporter plasmid pT7riboBUNMREN(-) is a derivative of pT7ribo (Dunn *et al.,* 1995). It expresses a negative-sense BUN M segment-like RNA under control of a T7 promoter, with the M segment coding sequence replaced by the *Renilla* (sea pansy) luciferase gene, and was constructed by a PCR-mediated approach (Dunn and Elliott, in preparation).

Production of T7-SIN cells, transfection, and dual reporter assays

T7 RNA polymerase-expressing BHK-21 cells were generated by transfecting *in vitro* transcribed RNA of the noncytopathic Sindbis virus replicon SINrep19 T7

(Agapov *et al.,* 1998), kindly provided by Charles M. Rice (Washington University School of Medicine, St. Louis, MO), and selecting for puromycin-resistant cell populations. These T7-SIN cells were maintained in Glasgow modified Eagle's medium (GMEM) supplemented with 10% newborn calf serum (NCS) containing 5 μg/ml puromycin (Sigma). For transfection with BUN plasmids, subconfluent monolayers of cells were incubated with plasmid DNAs in 500 μl OptiMEM (Gibco BRL) containing 15 μl liposomes (prepared as described by Rose *et al.,* 1991). After 5 h at 37°C, the liposome–DNA mixture was removed and replaced with 2 ml GMEM, and incubation continued for 18 h. The cells were then lysed in 200 μl of Dual Luciferase Passive Lysis Buffer (Promega), and 20 μl cell lysate was used to measure firefly and *Renilla* luciferase activities as described by the manufacturer (Promega).

Western blot analysis

Cells were lysed in Passive Lysis Buffer (Promega) and an aliquot containing 10 μg protein was separated on a 10% polyacrylamide gel by electrophoresis and blotted onto an Hybond-C membrane (Amersham) using a semidry transfer apparatus (Pharmacia). The membrane was incubated with a 1:500 dilution of rabbit anti-BUN N serum and the N protein band was visualised using the ECL method (Amersham).

Immunofluorescence analysis

T7-SIN cells were grown on coverslips to 30–50% confluency and transfected with 2 μg plasmid and 6 μl Fugene reagent (Roche) according to the manufacturer's instructions. Cells were fixed and permeabilised with ice-cold methanol at 18 h posttransfection. Cells were washed three times with phosphate-buffered saline (PBS) and incubated with the primary antibody, monoclonal mouse anti-FLAG M2 (Stratagene), diluted 1:500 in PBS containing 1% NCS. After incubation at room temperature for 1 h, the coverslips were washed three times in PBS and then treated with the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma), at a dilution of 1:200. Propidium iodide was included in the secondary antibody mix at a concentration of 1 μg/ml to stain the cell nuclei. Cells were again washed three times in PBS and mounted using AF1 glycerol/PBS solution (Citifluor). Stained cell samples were examined using a Zeiss Axiovert confocal laser scanning microscope with a X63 NA1.4 objective, linked to the LSM 510 software.

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